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(54) Title: IMMOBILIZED, POSITIONALLY NON-SPECIFIC LIPASE, ITS PRODUCTION AND USE

(57) Abstract

The immobilized lipase is positionally non-specific and more thermostable than those previously known. It is useful for ester hydrolysis, ester synthesis and interesterification processes. It may be produced by immobilization of lipase from *Pseudomonas cepacia.*

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Immobilized, positionally non-specific lipase, its production and use.

DEFINITIONS

The following definitions shall apply wherever the underlined words appear in this specification.

Lipase is taken to mean an enzyme that catalyzes reactions involving ester bonds (such as hydrolysis, synthesis and exhange of ester bonds) in water-insoluble carboxylic acid esters e.g. at an interface between aqueous and an organic phase.

Lipase may be in soluble, derivatized or immobilized form. Immobilized lipase denotes lipase in the form of immobilized enzyme or immobilized cells, as defined in "Guidelines for the characterization of immobilized biocatalysts" (1983), Enzyme Microb. Technol., 5, 304-307. Derivatized lipase denotes lipase that has been chemically modified without immobilizing it. Soluble lipase denotes un-modified lipase.

Lipases may be divided according to their positional specificity. As used in this specification, a positionally specific lipase (or specific lipase for short) is one that reacts only with the fatty acyl groups in the 1- and 3- positions of a triglyceride molecule, and a positionally non-specific lipase (or non-specific lipase for short) is one that reacts with all three fatty acyl groups of a triglyceride. Non-specific lipase activity can be determined in an interesteri-fication reaction with a triglyceride by measuring the reaction rate in the middle position, e.g. by reacting pure triolein or cocoa butter stearin with a pure fatty acid.

Lipase-catalyzed processes are ester hydrolysis, ester synthesis and interesterification. The esters involved may be triglycerides or other carboxylic acid esters.

In accordance with Bailey's Industrial Oil and Fat Products, Vol. 2, page 127, 4th Ed. (edited by D. Swern), interesterification refers to reactions in which a fat or other carboxylic acid ester is caused to react with carboxylic acids, alcohols, or other esters with the interchange of fatty acid groups to produce a new ester. Thus the reaction of an ester with an acid is called acidolysis, the reaction of an ester with an alcohol is called alcoholysis, and the reaction of one ester with another is termed ester interchange or transesterification.

Random interesterification of fats refers to interesterification reactions in which all three acyl groups in the triglycerides react, whereby a nearly random distribution of fatty acids in all three positions can be obtained. This may be achieved by use of a chemical catalyst or a non-specific lipase.

TECHNICAL FIELD

This invention relates to an immobilized, non-specific lipase preparation in particulate form, to a method for producing it, and to its use in a lipase-catalyzed process, i.e. interesterification, ester synthesis and ester hydrolysis. In particular, it relates to such an immobilized lipase with improved heat

BACKGROUND ART

Immobilized non-specific lipase is disclosed in Y. Kimura et al., Eur.J.Microbiol.Biotechnol. 17 (1983), 107 - 112. The lipase is derived from Candida cylindracea, and the data in the article show that the immobilized lipase has optimum temperature about 40°C, and that there is significant deactivation at 50°C.

Immobilized non-specific lipase and its use for random interesterification of fat are described in Macrae, A.R. (1983), Journal of the American Oil Chemists' Society (JAOCS), 60, 291. However, the process temperature was only 40°C. This low temperature was probably chosen due to the poor thermostability of the Candida cylindracea lipase.

Thus, prior-art preparations can only be used at about 40°C or lower. On the other hand, there is a need for thermostable, immobilized, non-specific lipase for processing high-melting substrates at about 60°C or higher without solvent, e.g. for randomization of fat in the margarine industry. Reference is made to A.R. Macrae and R.C. Hammond: "Present and Future Applications of Lipases", Biotechnology and Genetic Engineering Reviews, 3, 193,217 (1985).

Thus, it is the object of the invention to provide immobilized, non-specific lipase that is thermostable enough for long-term use at 60°C or higher. The lipase should be microbial, as these can be produced economically.

Thermostable immobilized specific lipase is known. As an example, European patent publication EP 0 140 542 (Novo Industri) discloses the use of such a product for interesterification at 70°C. But specific lipase does not fulfil the object of this invention.

Many non-specific microbial lipases are known in soluble form, and thermostability data have been published for the following: Staphylococcus aureus (Vadehra, D.V. (1974). Lipids, 9, 158), Penicillium cyclopium (Okumura, S., et al. (1976). Agricultural and Biological Chemistry, 40, 655 and Renshaw E.C. and San Clemente C.L. (1966) Developments in Industrial Microbiology, 8, 214), Corynebacterium acnes (Hassing, G.S. (1971). Biochimica et Biophysica Acta, 242, 381 and Pablo G. (1974) The Journal of Investigative Dermatology, 63, 231), Propionibacterium acnes (Ingham, E. et al. (1981). Journal of General Microbiology, 124, 393), Candida

cylindracea (also known as C. rugosa) (Benzonana, G. & Esposito, S. (1971). Biochimica et Biophysica Acta, 231, 15; and Kimura Y. (1983) Eur. J. Appl. Microbiol. Biotechnol., 17 , 107). Geotrichum candidum (Jensen R.G. (1974) Lipids, 9, 149; Jensen R.G. et al. (1972) Lipids, 7, 738; and Tsujisaka Y. and Iwai M. (1984) Kagaku to Kogyo, 58, 60). However, data in the literature referred to indicate that all these lipases have insufficient thermostability for long-term use at about 60°C or higher in immobilized form.

STATEMENT OF THE INVENTION

Surprisingly it has been found that positionally non-specific lipase can be obtained from Pseudomonas cepacia. And it has surprisingly been found that this lipase in immobilized form is heatstable enough for long-term use at 60°C, and is thus more heat-stable than previously known immobilized, non-specific lipases. The immobilized lipase is particularly suited for randomization of fat at temperatures as high as 60-80°C. Most fats are liquid at such temperatures and can be randomized without the presence of a solvent.

Lipases are known from Pseudomonas cepacia and from other species of Pseudomonas, but none of these are known to be positionally non-specific towards triglycerides. P. Eigtved et al. in a paper presented at the AOCS/JOCS Meeting in Honolulu on 1986-05-17 describe a Pseudomonas cepacia lipase that can esterify secondary alcohols (in contrast to most specific lipases), but is positionally specific in its action on triglycerides.

Accordingly, the first aspect of the invention provides an immobilized, positionally non-specific lipase preparation in particulate form, characterized in that the non-specific lipase is producible from Pseudomonas cepacia and/ or that it has immunological properties identical to

those of the non-specific lipase from the P. cepacia strain DSM 3959. This preparation has a half-life at 60°C above 1,000 hours when measured in continuous, fixed-bed interesterification.

Another aspect of the invention provides use of the above-mentioned immobilized preparation in a lipase-catalyzed process.

DETAILED DESCRIPTION OF THE INVENTION

Non-specific lipase

The group of non-specific lipases that can be used in the practice of the invention are those that are producible by a strain of Pseudomonas cepacia and/or have immunological properties identical to those of the non-specific lipase from P. cepacia strain DSM 3959. Immunological identity may be determined according to N.H. Axelsen et al. (ed.): Quantitative Immunoelectrophoresis, (Blackwell Scientific Publications, 1973), especially chapter 10 and to Ivan Roitt: Essential Immunology, 5th ed. (Blackwell Scientific Publications, 1984), especially Chapter 6.

Non-specific lipase for use in the invention may contain specific lipase as well, e.g. because the two lipases may be produced by the same microorganism. The content or absence of specific lipase is not critical to the practice of the invention.

Non-specific lipase for use in the invention may be produced by cultivating the strain DSM 3959 under aerobic conditions in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with principles known in the art.

The strain DSM 3959 was deposited at Deutsche Sammlung von Mikroorganismen (DSM) in West Germany on Jan. 30, 1987 under the terms of the Budapest Treaty. It has been identified as Pseudomonas cepacia.

The strain also produces specific lipase. An example of this specification demonstrates cultivation to produce non-specific lipase with a fairly low level of specific lipase, but at other conditions the ratio of specific to non-specific lipase increases.

Non-specific lipase for use in the invention may also be obtained according to Japanese published application 57-63,087 from the P. cepacia strain with deposit number 5494 at the Fermentation Research Institute, Japan. Specificity of the lipase preparation disclosed in said application has not previously been described, but we have found that the preparation contains non-specific lipase.

Genetic engineering techniques known in the art may be used to transfer the ability to produce lipase of the invention into other microbial strains. Use of such strains is also considered to be within the scope of the invention.

Immobilization method

For the practice of this invention, lipase may be immobilized by any method known in the art, e.g. in K. Mosbach (ed.): Methods in Enzymology, 44, "Immobilized Enzymes", (Academic Press, New York, 1976). Available methods for enzyme immobilization include: cross-linking of cell homogenates, covalent coupling to insoluble inorganic or organic carriers, entrapment in gels and adsorption on ion-exchange resins or other adsorbent materials. Also, coating on a particulate support may be used, as described in Macrae A.R. and Hammond R.C. (1985), Biotechnology and Genetic Engineering Reviews, 3, 193.

A preferred immobilization method uses a particulate, macroporous resin. The lipase may be simply adsorbed on the resin, or it may be attached to the resin by cross-linking with glutaraldehyde or other cross-linking agent known in the art.

A preferred resin type is weakly basic anion exchange resin, e.g. of acrylic, polystyrene or phenolic type. An example of a commercial product is Lewatit® E 1999/85 (product of Bayer, West Germany). The immobilization on this type of resin is preferably according to EP 0 140 542, incorporated herein by reference.

Another preferred resin type is an adsorbent resin of the phenol-formaldehyde type. The immobilization on this resin is preferably done according to DK 85/878, incorporated herein by reference.

Another preferred immobilization method uses an inorganic support material, and the lipase is preferably attached to the support by adsorption or covalent coupling. Such support materials and immobilization techniques are described in K. Mosbach (ed.): Methods in Enzymology, 44, "Immobilized Enzymes" (Academic Press, 1976).

Lipase-catalyzed processes

The lipase-catalyzed process of this invention may be any of the following types. In each case reactant types are listed in parentheses:

- Ester hydrolysis (ester + water)
- Ester synthesis (acid + alcohol)
- Interesterification, including:
    - Acidolysis (ester + acid)
    - Alcoholysis (ester + alcohol)
    - Ester interchange or transesterification (ester + ester)

The alcohol may be any mono- or polyvalent primary and/or secondary alcohol or a mixture of these. The acid may be any carboxylic acid or a mixture of these. The ester may be any ester derived from the mentioned alcohol and acid, or a mixture of these. Use of the immobilized lipase of the invention is particularly advantageous with triglycerides, where reaction in all three positions is desired.

Process temperature

The temperature in the process of this invention is preferably above 60°C, where most substrates and products of interest are liquid. Higher temperatures are generally preferred as reaction rate increases, and the diffusion resistance to mass transfer into and out of the immobilized lipase decreases. Also, in the case of column operation higher temperatures may be preferred to reduce the pressure drop over the column. On the other hand, in many cases the substrates and products will be degraded at the higher temperatures. Thus, a preferred range is 60-90°C, more preferably 60-80°C.

Ester hydrolysis process

Preferred embodiments of this process are fat splitting and hydrolysis of cholesterol esters. This may be performed either batch-wise or continuously.

In a batch reactor the fat and water are mixed mechanically together with the necessary amount of immobilized lipase. For reasons of economy in recovery, the water content will usually be kept below 40% w/w. The temperature should be above the melting point of the fat, and may be as high as 80°C. Reaction time depends on enzyme dosage and desired conversion, but may be up to several days. At the end of reaction, the immobilized lipase may be recovered and reused, thereby improving process economy.

In a continuous process, fat above its melting point is passed through a reactor in which the immobilized lipase is retained. Water may be added to the system in several ways, e.g. by dispersing water in the fat or by intermittently absorbing water in the immobilized lipase.

Ester synthesis process

The process of this invention is particularly advantageous for the synthesis of esters of secondary alcohols that are otherwise difficult to produce, including those where the acid or alcohol is high-melting.

The process may be performed batch-wise or continuously. In the batch process, the immobilized lipase may be recovered and reused to improve economy. Preferably, water is removed during reaction, e.g. by vacuum distillation or by absorption on molecular sieves. The temperature should be such that the reaction mixture is liquid, preferably 60-90°C, preferably 60-80°C.

Acidolysis

Preferably, the reactants comprise a triglyceride fat and a fatty acid.

Ester interchange

A preferred embodiment of this process is random interesterification of fat, where the reactant mixture comprises triglyceride fat, and reaction occurs by exchange of acyl groups between triglyceride molecules.

The reactant mixture may consist of a single fat fraction, whereby exchange between acyl groups in the three different positions occurs.

The reactant mixture may also consist of two or more types of fat, especially one being liquid at ambient temperature and one being a high-melting fat. The latter

may be obtained by fractionation from natural sources or by hydrogenation. The product obtained by randomization of such mixtures is useful in margarine production.

In another preferred embodiment of the ester interchange process, the reactants comprise a triglyceride fat and a carboxyl acid ester, especially a methyl or ethyl ester.

An organic solvent such as hexane or other hydrocarbons may be included in the reactant mixture. But in most cases it will be possible and preferable to run the process in melted fat without a solvent.

The reactant mixture may also include a small amount of water, in order to maintain the activity of the enzyme. Water content up to saturation may be used, but a high water content leads to an undesired high degree of by-product formation by hydrolysis.

Depending on the purity of reactants, purification may be needed prior to carrying out the reaction in order to achieve the highest productivity of the immobilized lipase. Conventional purification methods may be used, such as treatment with bleaching earth or activated carbon.

Due to the excellent thermostability of the lipase, reaction temperature may be as high as 80°C. The lower limit for reaction temperature is determined by the melting point and viscosity of the reactant mixture. Preferred temperatures are from 60 to 90°C, most preferably from 60 to 80°C.

The reaction may be performed batch-wise or continuously.

In the batch process the substrate and if convenient solvent is mixed in a batch reactor which is heated to the preferred temperature together with the immobilized lipase. The substrate can be partly or fully saturated with water. The enzyme dosage can be up to 10% depending on the desired conversion and reaction time. The

reaction time can be from a few hours to several days. After reaction the enzyme can be filtered off and reused, if convenient after a solvent wash.

In the continuous process the substrate is passed through a column containing the immobilized lipase. The substrate can if convenient be dissolved in hexane or similar inert solvents. The substrate can be partly or fully saturated with water before entering the enzyme column. This can e.g. be done by a precolumn containing water saturated resin or by saturating the substrate in the substrate container. The desired conversion can be achieved by adjusting the flow rate through the column, i.e. changing the contact time.

The operation time in such a system can be up to several thousand hours. The slow loss of activity occuring can be compensated for by decreasing the flow rate, i.e. increasing the residence time of the reactant mixture. A typical initial residence time will depend on desired conversion and can be from 5 min up to 2 hours.

After the interesterification, the products may be further processed. By-products such as free fatty acids may be removed afterwards by conventional methods such as caustic refining.

The product itself can be fractionated, blended with other oils or similar, depending on the specific application.

EXEMPLARY PRACTICE OF THE INVENTION

Assay for activity of soluble lipase

The method is based on hydrolysis of tributyrin in a pH-stat. 1 LU (Lipase Unit) is the amount of enzyme which liberates 1 µmol titratable butyric acid per minute at 30°C, pH 7.0 with gum arabic as an emulsifier. Further details are given in Novo Analytical Method AF 95/5, available on request.

Determination of Non-Specificity

In this method, positional specificity of immobilized lipase is determined by interesterification of cocoa butter stearin (which consists mainly of triglycerides of the XOX type, where O = oleic acid and X = palmitic or stearic acid) and lauric acid. The resulting triglycerides are converted to fatty acid methyl esters (FAME), and these are analyzed by gas chromatography (GLC). The decrease in oleic acid content expresses the non-specific lipase activity, and conveniently a Non-Specificity Index (NSI) may be calculated from the decrease of oleic acid and the total incorporation of lauric acid. NSI = 0 indicates absolute positional specificity, and NSI = 1 shows complete non-specificity.

More specifically, the non-specificity test is carried out as follows: The immobilized lipase (generally 250 mg as dry matter) is hydrated as required for activation usually to about 10% of water. The following mixture is used:

- 345 mg of cocoa butter stearin. (supplied by Aarhus Olie A/S, Denmark, and containing about 95% of SOS, POS and POP triglycerides: S= stearic acid, O= oleic acid, P= palmitic acid).
- 480 mg of lauric acid (Merck), 99% pure.
- 8.1 g of petroleum ether (BDH), boiling point 80-100°C.
- 250 mg (as dry matter) of the immobilized lipase.

A mixture of the above ingredients is incubated in a shaking water bath for a time and temperature (in the range 40-60°C) as needed to obtain a suitable conversion, as defined below. Pure triglycerides are then isolated by alumina chromatography, and fatty acid composition is determined by FAME-GLC, e.g. as described in Methods Ce 2-66 and Ce 1-62 published by the American Oil Chemists Society (AOCS). Conversion is considered suitable if the lauric acid content is 30-62 mole %. NSI is then calculated as:

$$NSI = 3 \times \frac{33.3 - \% O}{\% La}$$

where % O is mole % oleic acid, and % La is mole % lauric acid

EXAMPLE 1

Preparation of non-specific lipase from Pseudomonas cepacia

A culture of Pseudomonas cepacia strain DSM 3959 was transferred to a 500 ml shakeflask with 100 ml PSC-3 medium and shaken at 30°C for 1 day.

The composition of PSC-3 was as follows:

Glucose	3	g/l
$(NH_4)_2SO_4$	1	g/l
Na_2HPO_4	2.8	g/l
KH_2PO_4	4.2	g/l
Yeast extract	3	g/l

Autoclaved at 121°C for 20 minutes.

The resulting broth was used as seed culture for a 5 l conventional fermentor (Iwashiya mini jar fermentor AC-D-3) with 3 liters medium with the following composition:

Corn steep liquor	30	g/l
Glucose	6	g/l
KH_2PO_4	2	g/l
$(NH_4)_2HPO_4$	3	g/l
$MgSO_4 . 7H_2O$	1	g/l
Pluronic L60	1	g/l
pH	7.2	

After autoclaving the medium was inoculated with 30 ml seed culture and fermented 5 days with good agitation and aeration at 30°C with a continuous feed of 1.5 ml/hour soybean oil and 4.2 ml/hour 18.6% $(NH_4)_2SO_4$. The pH was controlled at 6.3 with carbonate.

The culture broth was concentrated to 800 ml by an Amicon Diaflow hollow fibre cartridge. One volume 96% ethanol was added to the concentrate, the mixture was stirred for 60 minutes at 4°C and centrifugated at 4200 g. The supernatant was freezedried, and the powder was dissolved in water to give 8000 LU/ml.

EXAMPLE 2

Immobilization of P. cepacia lipase

4.25 grams dry matter of Lewatit E 1999/85 weakly basic anion exchange resin (product of Bayer, West Germany) was adjusted to pH 7 and mixed with 12.5 ml of the lipase solution of Example 1. After rotation for 4 hours at room temperature the preparation was filtered, washed with water and dried in vacuum. The residual activity in the filtrate was 4500 LU corresponding to removal of 95% of the lipase activity and a load of 28,000 LU/g dry matter immobilized lipase.

The Non-Specificity Index was measured as described in the specification. In the table below fatty acid compositions are given in mole-% following reactions for 60 minutes at 60°C:

Lipase preparation	Fatty acid composition				NSI
	La	P	S	-Ol	
P. cepacia	60.5	10.1	14.9	14.5	0.93

It is seen that the lipase preparation is non-specific.

To determine activity of the immobilized preparation, 250 mg dry matter preparation was hydrated to 10% w/w, 600 mg triolein was added, 12 ml petroleum ether containing 174 mg palmitic acid was then added, the mixture was incubated for 12 min at 40°C, and incorporated palmitic acid (% w/w) was measured. From this figure, the activity was calculated as initial rate of palmitic acid incorporation, as follows:

Incorporated palmitic acid: 22.0 mole-%
Activity: 120 µmole/min/g

To determine heat-stability of the immobilized preparation, the activity measurement was repeated, but after addition of triolein the mixture was incubated at 70°C for 24 hours. Results as follows:

Incorporated palmitic acid:	18.6 mole-%
Residual activity:	77.2 µmole/min/g

It is seen that the preparation retains 64% of its activity after 24 hours at 70°C.

EXAMPLE 3

Randomization of fat

The ability of the P. cepacia immobilized non-specific lipase to randomize triglycerides (i.e. to mix the fatty acids to random positions in the triglycerides) was compared with a chemical method and with the interesterification by an immobilized 1,3 positional specific lipase. Cocoa Butter Stearin (CBS) (27.2 mole-% palmitic acid, 39.6 mole-% stearic acid, 31.3 mole-% oleic acid, 0.9 mole-% linoleic acid and 1.1 mole-% arachidic acid) was used as test substrate as it has the unsaturated fatty acid (oleic and linoleic) concentrated in the 2-position of the triglycerides.

The chemical randomization was carried out as follows: 3.0 g CBS was dried at 95°C for 30 minutes under vacuum by a rotary evaporator. Before addition of 30 mg sodium methoxylate ($NaOCH_3$) the temperature was reduced to 85°C and the vacuum was replaced with nitrogen at atmospheric pressure. The reaction was carried out for 1 1/2 hour under rotation before it was stopped by addition of 0.6 ml 1M HCl and washed 3 times with 5 ml deionized water at 60°C. The sample was dried at 95°C for 1 hour in the rotary evaporator before analysis.

The enzymatic interesterifications were carried out with immobilized P. cepacia lipase (prepared similarly to Example 2, but with a lipase load of 32,000 LU/g resin) and with Lipozyme™ IM20 (product of Novo Industri A/S), an immobilized, positionally specific lipase from Mucor miehei prepared according to EP 0 140 542. Both by the following procedure: 250 mg dry weight immobilized lipase was hydrated to 10% and 1.7 g CBS was added. The mixture was placed in a 70°C water bath and shaken for 1 hour. The reation was stopped by removing the enzyme by a filter.

The fatty acid composition in the 2-position of the triglycerides was analysed as follows: 100 mg CBS or interesterified CBS, 3 ml pancreatic lipase solution (250 mg porcine pancreas lipase grade II from Sigma cat. no. L3126 dissolved in 10 ml 1M trisbuffer pH 8), 300 µl 2M $CaCl_2$, and 0.75 ml 0.2% w/v taurocholate were mixed. The emulsion was heated in a water bath at 40°C for 2 minutes and mixed on a Whirley mixer for 1 1/2 minute before the reaction was stopped by addition of 4 ml 96% ethanol. The sample was transferred to a separation funnel and extracted with 4 x 20 ml diethyl ether. The ether fase was washed 4 times with 20 ml deionized water before it was dried by a Na_2SO_4 filter and evaporated. The sample was redissolved in 1 ml 1,1,1-trichloroethane. The glycerides were separated by preparative TLC on Precoated TLC Plates silica gel 60 from Merck (activated 30 minutes at 110°C) in a well saturated developing tank with diethylether-n-hexane (70:30) as developing solvent. The TLC was run 40 minutes at 20°C.

The monoglyceride band was identified by iodine vapour, scraped off and extracted by 3 times 10 ml diethyl ether. The ether fase was filtered, evaporated and the sample was methylated and analysed on GLC (procedure as described in AF 206/2 available from Novo Industri).

The results are presented below:

	Fatty acid composition of the 2-position	
	Saturated	Unsaturated
CBS before reaction	3.7 mole-%	96.3 mole-%
CBS chemically randomized	62.5 mole-%	37.5 mole-%
CBS reaction with *P. cepacia* lipase	65.6 mole-%	34.4 mole-%
CBS reaction with Lipozyme™ IM20	16.0 mole-%	84.0 mole-%
Equilibrium (= triglyceride composition)	66.8 mole-%	32.2 mole-%

Saturated fatty acids: palmitic acid, stearic acid and arachidic acid

Unsaturated fatty acid: oleic and linoleic acid

It is demonstrated that the *P. cepacia* interesterification, due to its activty towards the 2-position, approached a random distribution of fatty acid. The 1,3 positionally specific Lipozyme® 1M20 interesterification gives a very different composition as it leaves the 2-position largely untouched.

EXAMPLE 4

Continuous interesterification

4.5 g of the immobilized lipase of Example 2 was filled into a water jacketed column, having an internal diameter of 1.5 cm.

The column was heated by use of hot circulating water, i.e. 60°C. A precolumn containing water-saturated resin, (Duolite® ES561) was placed before the enzyme column and also heated to 60°C. A substrate consisting of 71% highly refined bleached and deoinized soy bean oil with a peroxide value less than 3 and 29% analytical grade lauric acid was pumped through the columns. At the outlet from the enzyme column samples were taken for analysis, and the incorporation of lauric acid measured by GLC. An incorporation of 14% w/w lauric acid was attempted and the flow rate was adjusted in order to keep the conversion at that value. Whenever the precolumn was dry it was replaced by a fresh one.

The samples were analysed by removing the free fatty acid and mono- and diglyceride by Al_2O_3-column chromatography, thereafter methylation of the triglyceride by $NaOCH_3$ and finally analysis of the methylester on a GLC.

The flow rate to obtain 14% La incorporation decreased with time, as follows:

Run time	Flow rate
239 hours	101.0 g/h
358 -	97.8 -
408 -	98.2 -

It is seen that very little deactivation occurs in 200 hours of operation, and the half-life (i.e. the time for the activity (or flow rate) to drop to half of the initial value) is estimated to be far above 1,000 hours.

CLAIMS

1. Immobilized, positionally non-specific lipase preparation in particulate form, characterized in that the non-specific lipase is producible by a strain of Pseudomonas cepacia, and/or that it has immunochemical properties identical to those of the non-specific lipase derived from Pseudomonas cepacia strain DSM 3959.

2. The preparation of Claim 1, characterized in that the non-specific lipase is obtainable from P. cepacia strain DSM 3959 or strain number 5494 from Fermentation Research Institute, Japan.

3. The preparation of Claims 1 - 2, wherein the lipase is immobilized on a particulate, macroporous resin, preferably by adsorption and/or cross-linking.

4. The preparation of Claim 3, wherein the resin is a weakly basic anion exchange resin.

5. The preparation of Claim 3, wherein the resin is an adsorbent resin, preferably of the phenol-formaldehyde type.

6. The preparation of Claim 3, wherein cross-linking is performed with glutaraldehyde.

7. The preparation of Claims 1 - 2, wherein the lipase is immobilized on an inorganic support material, preferably by covalent coupling or adsorption.

8. A method for producing an immobilized, positionally specific lipase in particulate form, characterized by comprising aerobically cultivating a non-specific lipase producing strain of Pseudomonas cepacia, or a transformant containing a gene encoding for and expressing the non-specific lipase thereof, in a suitable nutrient medium, thereafter recovering the non-specific lipase from the culture broth and immobilizing said lipase.

9. A method according to claim 8, comprising cultivation of Pseudomonas cepacia strain DSM 3959 or FRI 5494 or a transformant containing a gene encoding for and expressing the lipase thereof.

10. The immobilized lipase produced by the method of Claims 8 - 9.

11. Use of the preparation of Claims 1 - 7 or 10 in a lipase- catalyzed process.

12. Use according to Claim 11 in ester hydrolysis, preferably fat splitting or hydrolysis of cholesterol esters.

13. Use according to Claim 12 in a continuous process.

14. Use according to Claims 12 - 13, whereby the water content is below 40% w/w.

15. Use according to Claim 11 in ester synthesis.

16. Use according to Claim 15 in a batch process, where water is removed during the reaction.

17. Use according to Claim 15 - 16, whereby one reactant is a secondary alcohol.

18. Use according to Claims 15 - 17, whereby solvent is not used.

19. Use according to Claim 11 in interesterification, preferably without solvent.

20. Use according to Claim 19 in a continuous, fixed-bed process.

21. Use according to Claims 19 - 20 in acidolysis, preferably between triglyceride and a fatty acid.

22. Use according to Claims 19 - 20 in alcoholysis.

23. Use according to Claims 19 - 20 in ester interchange.

24. Use according to Claim 23 in random interesterfication of fat.

25. Use according to Claim 23, whereby the reactant mixture comprises triglyceride fat and a carboxylic acid ester, preferably a methyl or ethyl ester.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00125

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

C 12 N 9/20, C 12 N 11/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C 12 N 9/20; C 12 N 11/00, /08
US Cl	435:174,180,198

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above. Data base search: WPI, WPIL, CA.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, Vol 99 (1983), abstract No 69065m, Eur.J.Appl.Microbiol. Biotechnol. 1983, 17(2), 107-12 (Eng).	1-7
Y	Patent Abstracts of Japan, Vol 6, No 139, C-116, abstract of JP 57-63087, publ 1982-04-16, see the whole abstract	1-8,10-25
Y	EP, A3, 0 214 761 (NOVO INDUSTRI A/S) 18 March 1987 see abstracts and claims & JP, 62034997	1-8,10
Y	Chemical Abstracts, Vol 97 (1982) No 37532d, Jpn.Kokai Tokkyo Koho JP 82 63,087, 16 Apr 1982 see the whole abstract	1-8,10
Y	Chemical Abstracts, Vol 76 (1972), abstract No 70997y, Hakko Kogaku Zasshi 1971, 49(12), 968-80 (Eng). See the whole abstract.	1-10

.../...

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1988-10-18	1988 -10- 26
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Chemical Abstracts, Vol 108 (1988) abstract No 71297m, Res.Discl. 1987, 275, 121 (Eng). See the whole abstract	1-8,10,11,15
Y	EP, A1, 0 140 542 (NOVO INDUSTRI A/S) 8 May 1985 see page 3 lines 1-28, page 6 lines 14,18-19 & JP, 60098984 AU, 570720	1-8,10-14,25
P,Y	WO, A1, 88/02775 (NOVO INDUSTRI A/S) 21 April 1988	1-8,10-25